Diagnosis of Chronic Rejection

This invention relates to a method of monitoring the status of a transplanted tissue or organ in a recipient. In particular, the invention relates to the use of gene expression analysis to determine early prediction of chronic allograft rejection.

Chronic allograft rejection (CR) is the major cause for the failure of long-term graft survival. In contrast to treatable acute rejection episodes, chronic rejection is not reversible to date by any treatment when histologically detected, is not proven to be preventable by any immunosuppressive regimen and its pathogenesis is not fully understood but involving immunological as well as non-immunological factors. Characteristic for chronic rejection in all solid organ grafts is a concentric arterial intimal thickening by vascular remodeling. Kidney allografts with chronic rejection exhibit in addition pronounced parenchymal fibrosis and glomerular sclerosis: clinically, CR is manifested by a progressive decline in renal function, accompanied by proteinuria and hypertension.

Attempts to identify biomarkers in transplantation research have mainly been hypothesis-driven and lead to the characterization of individual genes, polymorphisms, biochemical or histopathological features. However, most of these studies were performed on tissue samples that already showed overt signs of disease and thus only revealed an association with the future severity of the disease.

There is a need to have a reliable tool for early prognosis and follow-up of CR, particularly early prognosis of CR before any overt clinical or histological manifestation, e.g. within the first year post transplantation; such an aid would be valuable e.g. for the optimisation of current treatment regimens and the design of clinical trials, including with new CR inhibiting agents.

The present invention relates to the identification of genes which are differentially expressed in transplant biopsies, e.g. renal biopsies, prior to the onset of CR in patients who will develop CR after the biopsy was taken, and patients that will not. The resulting gene expression pattern of a subset of the genes allows a highly statistically significant predictability of the occurrence of CR. For example, the genes identified in renal biopsies post transplantation before CR became histologically manifest, are indicated in Tables 1 (upregulated genes) and 2 (downregulated genes) and a subset of preferred genes in Table 3. The complete sequences of these 65 genes disclosed in this application are available

using the GenBank accession number shown in Tables 1 to 3. The sequences as shown under the corresponding GenBank accession number are incorporated herein by reference.

The genes identified according to the invention are useful predictive biomakers for the prognosis of CR in transplanted subjects. Any selection, of at least one, of these genes can be utilized as surrogate biomarker for early prognosis of CR. In particularly useful embodiments, a plurality of these genes, e.g. the 10 genes of Table 3 in case of renal biopsies, can be selected and their mRNA expression monitored simultaneoulsy to provide expression profiles for use in various aspects.

Accordingly, the invention provides the use of a gene as listed in Table 1, 2 or 3 as an early biomarker for chronic transplant rejection, e.g. as a biomarker for CR before any overt clinical or histological manifestation, e.g. within the first year post transplantation.

In a further aspect, the invention provides the use of a gene as listed in Table 1, 2 or 3, excluding FAS gene missing exon 4, retinoblastoma binding protein 7, prohibitin and connective tissue growth factor, as a biomarker for chronic transplant rejection.

In a further embodiment, the levels of the gene expression products (proteins) can be monitored in various body fluids, including, but not limited to, blood plasma, serum, lymph, urine, stool and bile, or in biopsy tissues. This expression product level can be used as surrogate markers for early diagnosis of CR and can provide indices of therapy responsiveness. An example is e.g. the protein encoded by the Connective Tissue Growth Factor (GenBank accession number X78947).

Accordingly, the invention also provides the use of an expression product of (e.g. a protein encoded by) a gene as listed in Table 1, 2 or 3 as an early biomarker for chronic transplant rejection, e.g. as a biomarker for CR before any overt clinical or histological manifestation, e.g. within the first year post transplantation.

In a further aspect, the invention provides the use of an expression product of (e.g. a protein encoded by) a gene as listed in Table 1, 2 or 3, excluding FAS gene missing exon 4, retinoblastoma binding protein 7, prohibitin and connective tissue growth factor, as a biomarker for chronic transplant rejection.

The methods of the present invention may be performed *in vitro*, e.g. the levels of biomarkers may be analysed in tissues or fluids extracted or obtained from a transplanted subject.

Methods of detecting the level of expression of mRNA are well-known in the art and include, but are not limited to, reverse transcription PCR, real time quantitative PCR, Northern blotting and other hybridization methods.

A particularly useful method for detecting the level of mRNA transcripts obtained from a plurality of the disclosed genes involves hybridization of labeled mRNA to an ordered array of oligonucleotides. Such a method allows the level of transcription of a plurality of these genes to be determined simultaneously to generate gene expression profiles or patterns. The gene expression profile derived from the biopsy obtained from the transplanted subject at risk of developing CR can be compared with the gene expression profile derived from the sample obtained from a transplanted subject that will not develop CR.

In a further embodiment, measuring expression profiles of one or a plurality of these genes or encoded proteins could provide valuable molecular tools for examining the efficacy of drugs for inhibiting, e.g. preventing or treating, CR. Changes in the expression profile from a baseline profile while the transplanted patient is exposed to therapy. Accordingly, this invention also provides a method for screening a transplanted subject to determine the likelihood that the subject will respond to the CR therapy, methods for the identification of agents that are useful in treating a transplanted subject having CR signs and methods for monitoring the efficacy of certain drug treatments for CR.

In one aspect, the invention features a (e.g. *in vitro*) method of identifying at least one gene which is differentially expressed in an allograft of a given tissue type prior to the onset of CR in a test transplanted subject compared to

i) a gene expression baseline profile originating from the same tissue type of a control transplanted subject who is known not to develop CR; or

ii) a gene expression baseline profile originating from the test transplanted subject at the date of the transplantation.

The term "differentially expressed" refers to a given allograft gene expression level and is defined as an amount which is substantially greater or less than the amount of the corresponding baseline expression level.

In another aspect, the invention provides a (e.g. in vitro) method of early diagnosing CR in a test transplanted subject by detecting a differentially expressed gene in a given allograft tissue sample. For example, the method may comprise

a) taking as a baseline value the level of mRNA expression corresponding to or protein encoded by at least one gene, e.g. as identified in Tables 1 and 2 or 3, the gene originating

from a specific allograft tissue biopsy of a control transplanted subject who is known not to develop CR;
b) detecting a level of mRNA expression corresponding to or protein encoded by the at least one gene identified in a) in an allograft tissue biopsy of the same tissue type as in a) obtained from a test transplanted subject within the first year post-transplantation; and
c) comparing the first value with the second value, wherein a first value lower or higher than the second value predicts that the test transplanted subject is at risk of developing CR.

According to another embodiment, the (e.g. in vitro) method may also comprise
a) detecting a level of mRNA expression corresponding to or protein encoded by at least one gene, e.g. as identified in Tables 1 and 2 or 3, in an allograft tissue biopsy obtained from the donor, preferably a living donor, at the day of transplantation,
b) detecting a level of mRNA expression corresponding to or protein encoded by the at least one gene identified in a) in an allograft tissue biopsy obtained from a patient within the first year post-transplantation,
c) comparing the first value with the second value, wherein a first value lower or higher than the second value predicts that the transplanted subject is at risk of developing CR.

In steps b) above, the level of mRNA or protein encoded is preferably detected within 4 to 7 months post-transplantation, more preferably around 6 months post-transplantation.

By prior to the onset of CR or early diagnosis of CR is meant before any overt clinical or histological manifestation of CR is detected in the transplanted subject.

The method of early diagnosing CR according to the invention may also be applied to maintenance patients, i.e. patients who have been transplanted more than one year ago. Accordingly, biopsies are performed and the level of mRNA expression corresponding to at least one gene is compared to the level in the reference control values to identify patients that will develop CR during the next couple of months.

In another aspect, the invention provides a method for monitoring, e.g. preventing or inhibiting or reducing or treating CR in a transplanted subject at risk of developing CR with a CR inhibitor (e.g. a small molecule, an antibody or other therapeutic agent or candidate agent). Monitoring the influence of agents (e.g. drug compounds) on the level of expression of a marker of the invention can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent to affect marker expression can be monitored in clinical trials of transplanted subjects receiving treatment for the inhibition of CR.

Such a method comprises:

a) obtaining a pre-administration sample from a transplanted subject prior to administration of the agent,

b) detecting the level of expression of mRNA corresponding to or protein encoded by the at least one gene in the pre-administration sample,

c) obtaining one or more post-administration samples from the transplanted patient,

d) detecting the level of expression of mRNA corresponding to or protein encoded by the at least one gene in the post-administration sample or samples,

e) comparing the level of expression of mRNA or protein encoded by the at least one gene in the pre-administration sample with the level of expression of mRNA or protein encoded by the at least one gene in the post-administration sample or samples, and

f) adjusting the agent accordingly.

For example, increased or decreased administration of the agent may be desirable to change the level of expression of the at least one gene to higher or lower levels than detected. In above method, the agent can also be administered alone or in combination with other agents in a combined therapy, preferably with immunosuppressive agents and/or agents effective in CR.

Accordingly, incorporation of gene expression profiling data from human biopsies, e.g. human renal protocol biopsies, will help improve the patient selection process during clinical trials aimed at both treatment and prevention of the progression towards CR.

In a yet other aspect, the invention further provides a method for identifying agents for use in the prevention, inhibition, reduction or treatment of CR comprising monitoring the level of mRNA expression of at least one gene or protein encoded as disclosed above.

In a further aspect, the invention provides a method for preventing, inhibiting, reducing or treating CR in a transplant subject in need of such treatment comprising administering to the subject a compound that modulates the synthesis, expression or activity of one or more genes or gene products, as disclosed in Tables 1, 2 or 3, so that at least one symptom of CR is ameliorated.

In a further aspect, the invention provides a compound (e.g. a small molecule, an antibody or other therapeutic agent or candidate agent) which modulates the synthesis, expression of activity of one or more genes or gene products identified above (e.g. a gene identified in

Table 1, 2 or 3) for use as a medicament, e.g. for the prevention or treatment of CR in a transplanted subject.

In a further aspect, the invention provides the use of a compound (e.g. a small molecule, an antibody or other therapeutic agent or candidate agent) which modulates the synthesis, expression of activity of one or more genes or gene products identified above (e.g. a gene identified in Table 1, 2 or 3) for prevention or treatment of CR in a transplanted subject.

In a further aspect, the invention provides the use of a compound (e.g. a small molecule, an antibody or other therapeutic agent or candidate agent) which modulates the synthesis, expression of activity of one or more genes or gene products identified above (e.g. a gene identified in Table 1, 2 or 3) for the preparation of a medicament for prevention or treatment of CR in a transplanted subject.

By transplanted subject is meant a subject receiving tissue or organ from a donor, preferably from the same species, e.g. kidney, heart, lung, combined heart and lung, liver, pancreas, bowel (e.g., colon, small intestine, duodenum), neuronal tissue, limbs.

Preferably more than one gene, e.g. a set of genes, are used in the methods of the invention. The methods of the invention are particularly preferred in kidney transplantation.

As already mentioned any selection, of at least one, of the genes indicated in Tables 1, 2 or 3 can be used. Preferably a selection of at least one gene of Table 1, e.g. ORP150 (oxygen regulated protein 150), thioredoxin, OS-9, NPRL2, HOXB7, G-CSF, BFGF etc and/or at least one gene of Table 2, e.g. prolactin receptor, etc. is used. Preferred genes of Table 3 are e.g.OS-9, NPRL2, HOXB7, G-CSF and/or BFGF. Most of the genes described here have not been implicated in renal allograft rejection and are associated with various functions. For example, OS9, also termed APRIL (acidic protein rich in leucines), is a member of the acidic nuclear phosphoprotein 32 family, with sequence homology to PHAPI, a putative HLA class II associated protein. OS9 also shares sequence similarity to the proto-oncogenes DEK and SET proteins, linked to myeloid leukemia. OBCML (opiate-binding protein/cell adhesion molecule-like) has only been described to be expressed in certain regions of the cerebellum, never in kidney. The tumor suppressor gene NPRL2, which shows sequence similarity to the yeast nitrogen permease regulator gene, has been shown to be located in the human chromosomal region 3p21.3, as one of 25 tumor suppressor genes within this region that are involved in the development of lung and breast cancer.

Gene expression profiles can be generated using e.g. the Affymetrix microarray technology. Microarrays are known in the art and consist of a surface to which probes that correspond in sequence to gene products (e.g. mRNAs, polypeptides, fragments thereof etc.). can be specifically hybridized or bound to a known position. Hybridization intensity data detected by the scanner are automatically acquired and processed by the GENECHIP^R software. Raw data is normalized to expression levels using a target intensity of 200.

The transcriptional state of a cell may be measured by other gene expression technologies known in the art. Several such technologies produce pools of restriction fragments of limited complexity for electrophoretic analysis, such as methods combining double restriction enzyme digestion with phasing primers (e.g. EP-A1-0 534858), or methods selecting restriction fragments with sites closest to a defined mRNA end (e.g. Prashar et al, Proc. Nat. Acad. Sci., 93, 659-663, 1996). Other methods statistically sample cDNA pools, such as by sequencing sufficient bases (e.g. 20-50 bases) in each multiple cDNAs to identify each cDNA, or by sequencing short tags (e.g. 9-10 bases) which are generated at known positions relative to a defined mRNA end (e.g. Velculescu, Science, 270, 484-487,1995) pathway pattern.

In another embodiment of the present invention, a protein corresponding to a marker is detected. A preferred agent for detecting a protein of the invention is e.g. an antibody capable of binding to the protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or preferably, monoclonal. An intact antibody or a fragment thereof (e.g. Fab or $F(ab')_2$ can be used. The term "labeled" is intended to encompass direct labelling of the antibody by coupling a detectable substance to antibody, as well as indirect labeling of the antibody by reactivity with another reagent that is directly labeled. A variety of formats can be employed to determine whether a sample contains a protein that binds to a given antibody. Examples of such formats include e.g. enzyme immunoassay, radioimmunoassay, Western blot analysis and ELISA.

In a preferred embodiment, the computation steps of the previous methods are implemented on a computer system or on one or more networked computer systems in order to provide a powerful and convenient facility for forming and testing models of biological systems. The computer system may be a single hardware platform comprising internal components and being linked to external components. The internal components of this computer system include processor element interconnected with main memory. The external components include mass data storage. This mass storage can be one or more hard disks. Other

external components include user interface device, which can be a monitor and keyboards, together with pointing device or other graphic input devices. Typically, the computer system is also linked to other local computer systems, remote computer systems or wide area communication networks, e.g. Internet. This network link allows the computer system to share data and processing tasks with other computer systems.

Loaded into memory during operation of this system are several software components which are both standard in the art and special to the instant invention. These software components collectively cause the computer system to function according to the methods of this invention. These software components are typically stored on mass storage or on removable media, e.g. floppy disks or CD-ROM. The software component represents the operating system, which is responsible for managing the computer system and its network interconnections. preferably the methods of this invention are programmed in mathematical software packages, which allow symbolic entry of equations and high-level specification of processing, including algorithms to be used, and thereby freeing a user of the need to procedurally program individual equations or algorithms.

In preferred embodiments, the analytic software component actually comprises separate software components that interact with each other. Analytic software represents a database containing all data necessary for the operation of the system. Such data will generally include, but is not limited to, results of prior experiments, genome data, experimental procedures and cost, and other information, which will be apparent to those skilled in the art. Analytic software includes a data reduction and computation component comprising one or more programs which execute the analytic methods of the invention. Analytic software also includes a user interface which provides a user of the computer system with control and input of test network models and, optionally, experimental data. The user interface may comprise a drag-and-drop interface for specifying hypotheses to the system. The user interface may also comprise means for loading experimental data from the mass storage component, from removable media or from a different computer system communicating with the instant system over a network.

The invention also provides a process for preparing a database comprising at least one of the markers set forth in this invention, e.g. mRNAs. For example, the polynucleotide sequences are stored in a digital storage medium such that a data processing system for standardized representation of the genes that identify early prognosis of CR. The data processing system is useful to analyze gene expression between two tissue samples taken

at different time point, e.g. at the transplantation day and post- transplantation. The isolated polynucleotides are sequenced. The sequences from the samples may be compared with the sequence(s) present in the database using homology search techniques. Alternative computer systems and methods for implementing the analytic methods of this invention will be apparent to one skilled in the art and are intended to be comprehended within the accompanying claims.

Identification of Prognostic Markers of CR

As a part of a randomized, multicenter, double-blind, double-dummy, parallel group study, serial renal protocol biopsies are taken at the time of transplantation (baseline), then 6 months and 12 months after transplantation.

After RNA extraction from these biopsies, the overall yield of total RNA ranges from only around 10 ng to around 1,5 µg. Most of the samples contain less than 30 ng total RNA, which is far below the minimal amount of 1 to 5 µg that commercially available RNA labeling kits and methods need for subsequent microarray experiments. In order to obtain sufficient amounts of RNA for microarray experiments, linear RNA amplification is performed. Briefly, this method involves subsequent rounds of cDNA and aRNA synthesis, which amplifies the original amount 20-30 fold per round. 90 RNA samples are amplified and labeled. aRNAs are hybridized to Affymetrix HG U95A v2 chip containing oligonucleotide probes of about 12,000 human genes and analyzed.

Tissue homogenization

All liquid nitrogen flash-frozen biopsy samples are stored in cryotubes at -80°C. Immediately after the addition of 700µl homogenization buffer (ABI lysis buffer/PBS 1:1) the homogenization step is performed by dipping the rod of a Polytron rotor/stator homogenizer PT 3100 into the tissue containing buffer and running the homogenizer at full speed for 30 seconds. If after this time remnant tissue pieces are visible, the procedure is repeated until homogenicity is achieved. Hereafter the homogenate is stored at -80°C until it is used in the RNA extraction step.

Homogenate pre-filtration and RNA extraction

Pre-filtration of the homogenate and RNA extractions are performed by the ABI 6700 Biorobot workstation (Applied Biosystems, USA). Tissue homogenates are filled into the wells of a 96-deep-well plate, and placed in the filtrate position of the 6700 workstation. A tissue pre-filter tray is placed into the purification carriage and locked into position. The instrument door is closed, and the workstation software is launched.

The RNA extraction procedure includes a sample transfer step, a filtration step, a washing step, and an elution step. The sample transfer step, in which the pre-filtered homogenate is transferred from the 96 deep-well plate to the RNA purification tray includes a primary transfer of 550 µl solution. Before the second transfer, 150 µl homogenization buffer (Applied Biosystems lysis buffer/PBS 1:1) is added to each well in the deep-well plate, mixed three times and then 150 µl are transferred from there to the purification tray. The filtration step is carried out by applying a vacuum pressure of 80% for 180 seconds. The washing steps are performed as follows:

Step 1: washing solution 1, 400 µl, vacuum pressure 80% for 180 seconds, two times;

Step 2: washing solution 2, 500 µl, vacuum pressure 80% for 180 seconds, once;

Step 3: washing solution 2, 300 µl, vacuum pressure 60% for 120 seconds, two times.

A pre-elution vacuum of 90% pressure is applied for 300 seconds. Hereafter the elution step is performed by the addition of 120 µl elution solution (Applied Biosystems), and the application of a 100% vacuum-pressure for 120 seconds. The RNA samples are collected in 96-well plates (Applied Biosystems). The eluates are split into two aliquots of equal volume. One aliquot is stored at - 80°C, the other aliquot is used for RNA amplification and GeneChip analysis.

RNA amplification

Prior to the RNA amplification procedure, all RNA eluates are treated with RNeasy kit chemistry (Qiagen) to further clean the RNA from remnant salt or other substances that may inhibit the amplification efficiency. The volume of the aliquot is adjusted to 100 µl with RNase-free water. 350 µl buffer RLT is added and mixed thoroughly. 250 µl ethanol (96-100%) is added, and mixed thoroughly. The sample (700 µl) is applied to an RNeasy mini column, placed in a 2 ml collection tube. After a 15 second centrifugation step at more than 10,000 rpm, the flow-through is discarded. The RNeasy column is transferred into a new 2 ml collection tube. 500 µl buffer RPE is pipetted onto the column, the tube closed and centrifuged for 15 seconds at more than 10,000 rpm to wash the column. The flow-through is discarded. Another 500 µl buffer RPE is added to the column and the tube is centrifuged for 2 minutes at more than 10,000 rpm to dry the silica-gel membrane. To elute, the RNeasy column is transferred to a new 1,5 ml collection tube and 30 µl RNase-free water is added directly onto the membrane. After 1 minute incubation, the tube is centrifuged for 1 minute at more than 10,000 rpm to elute. This elution step is repeated once to get a total elution volume of 60 µl. The RNA is quantified by the Ribogreen method (Molecular Probes, Inc, USA). About 10 ng total RNA of each sample is used in three rounds of RNA amplification.

All enzymes and buffers for the amplification procedure are purchased from Invitrogen, Inc. (Carlsbad, CA, USA) unless explicitly mentioned. 10ml total RNA are incubated with 10 pmol T7-polydT primer [5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$(T)_{24}$] (Genset, Inc.) in a volume of 11µl at 70°C for 10 minutes, then at 42°C for 5 minutes. The first strand reaction is carried out in a volume of 20µl by the addition of 200 units SuperScript II in the presence of first strand buffer, 10 mM DTT, 0.5 mM dNTP mixture, and 1µl RNase inhibitor (Ambion, Inc.) with a 42°C-incubation for 1hr. The second strand synthesis is performed in 150 µl with 40 units E.coli DNA polymerase I in 1x second strand buffer, 0.2 mM dNTPs, 10 units E.coli DNA ligase and 2 units RNaseH. After a 2-hour incubation at 16°C, the double-stranded DNA is blunt-ended by the addition of 8 units T4 DNA polymerase for 10 minutes at 16°C. The double-stranded DNA product is purified with a QIAquick PCR purification kit (Qiagen) and eluted in 50 µl elution buffer. For only one round of amplification the volume of the eluate is reduced to dryness under vacuum, resuspended in 22 µl nuclease-free water, and then used in the RNA labelling reaction as described below. For additional rounds of amplification, the eluate is reduced to dryness under vacuum, resuspended in 8 µl nuclease-free water, and subjected to an in-vitro transcription reaction with the Ambion MEGAscript kit, following the manufacturer's instructions for a 20 µl reaction volume. After a 3-hour incubation at 37°C the RNA is purified with the RNeasy kit system (Qiagen). The RNA is eluted in 30 µl RNase free water, reduced to dryness under vacuum and resuspended in 11 µl nuclease-free water.

The second round of RNA amplification started with the addition of 1µl 0.1 mg/ml random hexamer primers followed by a 10-minute incubation at 70°C. The reaction mixture is chilled on ice and then incubated at room temperature for 10 minutes, at which point the first strand synthesis reaction is started by the addition of 200 units SuperScript II, 20 units RNase Inhibitor, 0.5 mM dNTPs and 10mM DTT in the presence of first strand reaction buffer. The mixture is incubated at 37°C for 1hour. A 20-minute RNase H treatment (2 units) at 37°C lead to the degradation of the residual RNA. RNase H is heat-inactivated at 94°C for 2 minutes and the mixture is chilled on ice. The second-strand synthesis is initiated by the addition of 100 pmol T7-polydT primer (see above) and incubation at 70°C for 5 minutes, followed by 42°C for 10 minutes. The second-strand synthesis is performed as described above, and the cDNA is purified with the QIAquick PCR purification kit (Qiagen). If this is the final round of amplification, the volume of the eluate is reduced to dryness under vacuum and resuspended in 22 µl nuclease-free water, followed by an in-vitro RNA labelling procedure (see below).

A third round of amplification is prepared by reducing the eluate to dryness under vacuum and resuspended in 8 µl nuclease-free water before it is then subjected to the procedure identical to that of a second round of amplification. We observed a 15 to 30-fold increase in aRNA in each round of amplification, resulting in $5x10^5$ to $1.9x10^6$-fold amplification of messenger RNA after three rounds (assuming 3.3 % poly(A)+ RNA within the initial pool of total RNA). Labelled RNAs are fractionated at 94°C for 35, 25, or 20 minutes for single (1x), double (2x), or triple (3x) amplified RNAs, respectively. Shorter incubation times for 2x RNA and 3x RNA are chosen to avoid complete degradation of the RNA.

The RNA biotinylation step involved the use of the High-Yield RNA Labelling Kit (Enzo Diagnostics, NY, USA; P/N 900182) following the manufacturer's instructions. The following ingredients are mixed in an initial step:

22 µl aRNA

4 µl 10X HY reaction buffer,

4 µl 10X Biotin Labelled Ribonucleotides,

4 µl 10X DTT,

4 µl RNase inhibitor mix,

2 µl 20X T7 RNA polymerase.

The mixture is incubated at 37°C for 3-4 hours. The labelled aRNA is purified using RNeasy chemistry (Qiagen) following the manufacturer's instructions.The elution volume is 60 µl, 2 µl are used to determine the RNA concentration spectrophotometically by absorbance at 260 nm.

RNA fragmentation

15 µg labelled aRNA is fragmented in a volume of 20 µl by the addition of 4 µl 5X MES Fragmentation buffer and RNase free water. The mixture is incubated for 20 minutes at 94°C.

12X MES Fragmentation buffer (for 1000ml):

70,4 g MES free acid

(1,22M MES, 0.89M [Na^+] (2-(N-Morpholino)ethanesulfonic acid (SIGMA, P/N M5287)

193,3 g MES sodium salt (Sigma, P/N M3885)

800 ml DEPC water

Filter through a 0.2 µm filter, the pH should be between 6.5 and 6.7 without adjustment.

Microarray hybridization mix

The hybridization is carried out in a volume of 300 µl. Fragmented aRNA is mixed with 150 µl 2X MES hybridization buffer, 3 µl herring sperm DNA (10mg/ml), 3 µl BSA (50mg/ml), 3 µl

948b control oligonucleotide (5nM), and 3 µl 20X Eukaryotic Hybridization Controls (Affymetrix). DEPC water is added to 300 µl final volume.

2X MES Hybridization buffer (for 500 ml):

217 ml DEPC water

200 ml 5M NaCl

82 ml 12X MES

Filter through 0.2 µm filter.

Then add: 1.0 ml 10% Triton X-100. Store at room temperature.

Microarray pre-treatment

The microarray is incubated at 45°C for 15 minutes. The array chamber is filled with freshly prepared pre-treatment solution, prewarmed to 45°C.

Pre-treatment solution (300µl per microarray)

294 µl 1X MES hybridization buffer

3 µl Acetylated BSA (50mg/ml) (Gibco BRL Life Technologies, P/N 15561-020)

3 µl Herring sperm DNA (10mg/ml) (Promega/Fisher scientific, P/N D1811)

Microarray hybridization

While the microarrays are being pre-treated at 45°C, the hybridization mix is incubated at 99°C for 5 minutes. After a centrifugation for 5 minutes at 14,000 rpm the supernatant is transferred to a new Eppendorf tube and incubated at 45°C for 5 minutes. The pre-treatment solution is removed from the microarray chamber and replaced with the hybridization mix, avoiding bubbles. The septa of the plastic cartridge are covered with tape and the cartridge is placed in an oven at 45°C with the glass front facing down. The hybridization is continued for 16 to 18 hours.

Washing Procedure

The hybridization mix is removed from the probe array and set aside in a microcentrifuge tube. 280 µl 1X MES hybridization buffer is added to the chamber and a fluidics wash is performed on a GeneChip Fluidics Station 400 using 6X SSPE-T buffer.

6X SSPE-T wash buffer (1000ml)

300 ml 20X SSPE (BioWhittaker, P/N 16-010Y)

699 ml water

Filter through 0.2 µm filter. Add 1 ml 10% Triton X-100

After the fluidics wash the SSPE-T buffer is removed from the chamber and filled with stringent wash buffer, avoiding bubbles.

Stringent wash buffer (1000ml):

83.3 ml 12X MES buffer

5.2 ml 5M NaCl

1 ml 10% Tween 20

910.5 ml water

Filter through 0.2 µm filter. Add 1ml 10% Triton X-100.

The microarray cartridges are layed face up in a 45°C incubation oven for 30 minutes. The stringent buffer is removed and the array is rinsed with 200 µl 1X MES hybridization buffer. The 1X MES hybridization buffer is completely removed, the array chamber filled with SAPE stain, and incubated at 37°C for 15 minutes.

SAPE stain (600 µl):

300 µl 2x MES hybridization buffer

288 µl water

6 µl BAS (50mg/ml)

6 µl SAPE (1mg/ml) (Molecular probes, P/N 15230-147)

After 15 minutes the SAPE stain solution is removed, the chamber filled with 200 µl 1X MES hybridization buffer, and a fluidics wash is performed. The SSPE-T solution is removed from the microrarray chamber and replaced with 300 µl AB stain.

AB stain (300 µl):

150 µl 2X MES hybridization buffer

146.25 µl water

3 µl BSA (50 mg/ml)

0.75 µl biotinylated antibody (500µg/ml) (Vector laboratories, P/N BA-0500)

The cartridge is incubated at 37°C for 30 minutes, the AB stain is replaced with 200 µl 1X MES hybridization buffer, and a fluidics wash is performed. After the wash step, the SSPE-T solution is removed, the chamber is filled with SAPE stain, and incubated at 37°C for 15 minutes. The SAPE stain is replaced with 200 µl 1X MES hybridization buffer and a fluidics wash is performed. The septa are covered with tape to prevent buffer leakage.

Microarrays are scanned on Affymetrix GeneArray® scanners. Raw data sets are normalized by scaling 75%- quantile of all probe sets of each chip to a target intensity of 200.

Separation Method

Statistical analysis is performed with S-Plus (Insightful, Inc., USA) and GeneSpring^{R} (Silicon Genetics, USA). Average difference values of less than 10 are rounded to 10. Low expression levels (between 10 and 50) are kept to ensure not to lose any possible pattern.

Standard parametric and non-parametric statistics (Student's t-test, Wilcoxon's rank sum test) are applied.

More particularly, an algorithm is developed to separate the data for a particular gene for the CR and control group, a distinction being made between the CR group which has lower expression values than the control group and the CR group which has higher expression values than the control group. In addition to having separate expression ranges, the separation gap between the groups is maximized. Thus the gap between a specified quantile (Q) of the 2 data sets (i.e the 1-α quantile of the group with lower expression levels and α quantile of the group with higher expression levels with $0<\alpha<0.5$).

The statistic to measure how well each gene separates the two groups is given as

$$S = \max (q_{\alpha, cr} - q_{1-\alpha, control}, q_{\alpha, control} - q_{1-\alpha,cr}, 0)$$

where $q_{\alpha, cr}$ denotes the α quantile of the CR group with $0 < \alpha < 0.5$ (and therefore $1 - \alpha > 0.5$). Similarly, control or cr denotes the control group.

The 0 is added to the evaluation of S because both terms, $q_{\alpha, cr} - q_{1-\alpha, control}$, and $q_{\alpha, control} - q_{1-\alpha, cr}$, can be negative. In this case, measuring the difference does not make any sense, as the groups overlay too much. In other words, the 1-α quantile of the one group must be below the α quantile of the other.

An algorithm with following settings is used:

- Threshold (for noise) set to T = 10.
- Threshold (for minimum expression) set to $T_2 = 50$.
- The minimum percentage of signals above threshold T_2 is set in *each* group to P = 40%.
- The minimum percentage of signals above threshold T_2 over all groups combined is set to P = 50% (such that a higher percentage in one group can to some extent compensate a lower percentage in the other group).
- The percentage of signals below T_2 is determined for each gene, individually for each group (CR, control).
- All genes that do not fulfill all minimum percentage thresholds are discarded.
- The statistic S is calculated for each gene.
- The genes with S > 0 are returned, sorted by S.

As a first pass filter all genes containing a too large proportion of low signals are excluded. A gene is included in the dataset if

- both groups, CR and control, contain at least 40 percent values above the threshold T_2 = 50 (at least 4 out of 8 and 4 out of 9 samples).
- the combined dataset contains at least 50 percent values above the threshold T = 10 (at least 9 out of 17 samples).

In a second pass, all expression values below 10 are set to 10 to reduce random variation. (The Affymetrix algorithm used to derive the expression levels can generate negative values). Low levels (between 10 and 50) are preferred to ensure not to lose any pattern that might occur.

In GeneSpring™, each gene is normalized to itself by creating a synthetic positive control for that gene, which is the mean of all values of that gene in a dataset, and dividing all measurements for that gene by this positive control, assuming it is at least 0.01.

TaqMan Primer Probe Design

TaqMan assays should be designed to the region of a gene that hybridizes to the corresponding probe set of the HG-U95Av2 microarray. This region is called target sequence. Using the Netaffx™ software (Affymetrix, Inc), the target sequence of a probe set is identified. The target sequence is then imported into the program Primer Express (Applied Biosystems), and the primer/probe selection is performed by the program with the following conditions:

Primer TM (melting temperature) should be between 58°C and 60°C. Optimally it should be 59°C, with a maximum TM difference of 2°C.

The primer GC (GTP,CTP) content should be between 20% at the minimum and 80% at the maximum, avoiding any 3' GC clamps.

The optimal primer length should be 20 residues, but can range form 9 to 40 residues.

The amplicon requirements should be that the minimum TM is 0°C, the maximum TM 92°C. The amplicon should have a minimal length of 50 residues, a maximal length of 150.

TaqMan probe criteria are that the probe TM must be at least 0°C greater than the PCR primer TM, and the probe should not begin with a G (GTP) residue. If the target sequence is too short to identify any TaqMan assay matching the above mentioned criteria, the sequence is aligned to the entire sequence of the gene (using standard software such as GCG, Wisconsin Package, Accelrys, San Diego, CA) and a longer stretch of DNA is selected, encompassing the target sequence. Sequences of the forward primer, reverse primer and the TaqMan probe for each gene are listed in Table 4.

To identify the genes that separate best the chronic rejection group from the control group standard techniques are applied, e.g. t and Wilcoxon statistics, and a newly derived measure to find well-separated groups (with a large separation gap in between) is included.
The measure (Q15/85 and Q20/80) extends the concept of separation to finding a good separation gap. It is based on measuring the distance between the α-quantile of the CR group and the $(1-\alpha)$-quantile of the control group and vice versa. If the $(\alpha, 1-\alpha)$ ranges of both groups do not overlap, the maximum distance is reported, otherwise 0. Applying the measures to each gene individually delivers a measure for the separation of the 2 groups. The Q20/80 method identified 65 genes and the Q15/85 method 16 genes with complete separation of the (20%, 80%) and (15%, 85%) quantile ranges, respectively.

Comparing the genes detected to the ordered t- and Wilcoxon statistic identified that 10 of those genes are ranked among the 100 with most extreme t- *and* Wilcoxon statistic. The gene identifiers and annotations are given e.g. in table 3.

Almost all genes identified show expression levels above the low threshold of 50, mostly even much higher.

Applying a different way of evaluating the power of a set of genes to separate the two groups of samples, a cluster analysis of all 65 genes that are common to both, the t statistic list and the Wilcoxon list is performed. The cluster is prepared in two steps. First, a gene cluster is prepared by standard correlation, then an array cluster by Pearson correlation. To make a tree, GeneSpring calculates the correlation for each gene with every other gene in the set. Then it takes the highest correlation and pairs those two genes, averaging their expression profiles. GeneSpring then compares this new composite gene with all of the other unpaired genes. This is repeated until all of the genes have been paired. At this point the minimum distance and the separation ratio come into play. Both of these affect the branching behavior of the tree. The minimum distance deals with how far down the tree discrete branches are depicted. The number specified in the minimum distance box determines the minimum separation considered significant between genes. This reduces meaningless structure at the base of the tree. Decreasing minimum distance increases the 'branchiness' of the tree. Default minimum distance is 0.001. A value smaller than 0.001 has very little effect, because most genes are not correlated more closely than that. A higher number will tend to lump together more genes into a group, making the groups less specific.

This number should be between 0 and 1. The separation ratio determines how large the correlation difference between groups of clustered genes has to be for the groups to be

considered discrete groups and not be joined together. Increasing separation increases the 'branchiness' of the tree. The default separation ratio is 0.5, but it can range from 0.0 to 1.0. At a separation ratio of 0, all gene expression profiles can be regarded as identical.

The Pearson correlation is very similar to the Standard correlation, except it measures the angle of expression vectors for genes A and B around the mean of the expression vectors (for example, the mean of the expression values constituting the profiles for Gene A and Gene B). Generally the mean of the expression vectors will be positive since expression values are based on concentrations of mRNA. Using the Pearson correlation more negative correlations are obtained than from the Standard correlation. It is worth noting that the Pearson correlation gives you almost the same correlations as the Standard correlation when they are both performed on the logarithms of the genes' expression values. This is how to compute a Pearson correlation: calculate the mean of all elements in vector a. Then subtract that value from each element in a. Call the resulting vector A. Do the same for b to make a vector B. Pearson Correlation = A.B / (I A II B I)

The gene cluster is prepared by performing a standard correlation analysis with a separation ratio value of 0.5 and a minimum distance value of 0.001. For the microarray cluster a Pearson correlation is used with a separation ratio of 1.0 and a minimum distance value of 0.001. Accordingly, the set of 65 genes separates the two patient groups perfectly. The distance between the two groups is rather small. This was expected since all patients were clinically and histologically healthy at the point these biopsies were drawn.

By applying above method the expression levels of about 12,000 transcripts in serial renal allograft protocol biopsies from 17 transplant patients have been monitored. Demographic and clinical characteristics of all patients in this study are listed in Table 5. One group of patients developed CR within 6 months after the timepoint the biopsy was taken, the other group did not. Using the set of genes as disclosed, preferably the set of 10 genes as indicated in Table 3, as identified by detailed statistical analysis of the biopsy RNA expression profiles, the occurrence/non occurrence of chronic rejection was predicted in 15 out of these 17 patients (> 88 %). Furthermore, the set of discriminator genes was also able to predict that a month 12 biopsy belonged to a patient that developed CR until month 18.

Table 1: List of genes (with GenBank accession numbers) which are upregulated in pre-CR group

GenBank accession number	Affymetrix probe set	description	fold change	Wilcoxon p-value	t-test p-value
X07696	37582_at	cytokeratin 15 / KRT15	7.96	0.0003	0.0192
AB005666	36843_at	GTPase-activating protein	6.93	0.0064	0.0284
U65785	33863_at	ORP150 mRNA	6.54	0.0193	0.0051
M16937	37618_at	homeobox c1 protein / hoxB7	5.73	0.0009	0.0197
Z37166	35292_at	BAT1 mRNA (DEAD family)	5.50	0.0266	0.0163
U48231	33549_at	bradykinin B1 receptor	4.88	0.0030	0.0108
U77968	34652_at	member of the bHLH-PAS family	4.55	0.0047	0.0059
AJ000480	35597_at	C8FW phosphoprotein	4.30	0.0046	0.0089
AF040708	40499_r_at	NPRL2 / similar to yeast NPR2 nitrogen permease	4.28	0.0041	0.0058
AJ011497	38482_at	claudin-7	4.34	0.0193	0.0044
Z70519	37644_s_at	FAS gene missing exon 4	4.41	0.0064	0.0103
U41635	36996_at	OS-9	2.70	0.0011	0.0388
U45448	33535_at	ATP-gated ion channel	2.69	0.0104	0.0167
AB010414	39893_at	G-protein gamma 7	2.54	0.0030	0.0186
X98411	35132_at	myosin-IF	2.47	0.0152	0.0240
AB011082	36408_at	ORCTL4	2.38	0.0289	0.0230
X63546	1613_s_at	tre oncogene	3.05	0.0068	0.0048
S80864	35955_at	putative protein	3.01	0.0289	0.0189
AI827895	36224_g_at	IMAGE:2350347	2.98	0.0152	0.0406
U13897	40246_at	human homolog of Drosophila discs large protein	2.88	0.0071	0.0072
U16799	37669_s_at	Na,K-ATPase beta-1 subunit	2.88	0.0211	0.0163
X14445	1855_at	int-2 (FGF-3)	2.92	0.0107	0.0406
AI653621	36992_at	thioredoxin	3.12	0.0101	0.0108
U79251	41093_at	OBCML, opioid binding protein/cell adhesion molecule-like	3.48	0.0047	0.0166
M59818	34223_at	granulocyte colony-stimulating factor receptor (G-CSFR-1)	3.58	0.0095	0.0310
D11086	1506_at	interleukin 2 receptor gamma chain	3.71	0.0149	0.0146
NM002893	1515_at	retinoblastoma binding protein 7	4.01	0.0106	0.0281
AL049449	33997_at	DKFZp586B1722	4.08	0.0107	0.0333
AL049228	31985_at	DKFZp564N1716	4.10	0.0249	0.0228
X64116	32698_at	poliovirus receptor	1.92	0.0107	0.0134
S85655	36592_at	prohibitin	1.94	0.0152	0.0098
W26469	31377_r_at	EST ID 32f4 from retina cDNA randomly primed sublibrary	2.23	0.0152	0.0208
AB020638	33226_at	KIAA0876	2.25	0.0152	0.0402

Table 2: List of genes (with GenBank accession numbers) early downregulated in pre-CR group

GenBank accession number	Affymetrix probe set	description	fold change	Wilcoxon p-value	t-test p-value
U56637	40910_at	capping protein alpha subunit isoform 1	4.16	0.0010	0.0036
D14110	1276_g_at	RNA binding protein	3.76	0.0030	0.0135
M24194	34609_g_at	homologue; putative (G-protein)	3.71	0.0046	0.0168
X56777	39720_g_at	ZP3	3.48	0.0095	0.0301
U94747	38171_at	WD repeat protein; similar to petunia AN11	3.49	0.0172	0.0282
AB018313	39130_at	KIAA0770	3.47	0.0211	0.0469
U66059	32795_at	T cell receptor beta locus	3.30	0.0203	0.0410
M93651	40189_at	SET gene	5.97	0.0028	0.0141
AJ005814	40343_at	hoxA7	5.03	0.0055	0.0549
X75861	33988_at	TEGT	5.27	0.0071	0.0470
X78947	36638_at	connective tissue growth factor	5.22	0.0208	0.0489
L05095	31708_at	ribosomal protein L30	10.83	0.0211	0.0579
U52112	31873_at	ARD1 subunit homolog	3.01	0.0046	0.0106
AF031416	35960_at	IKK beta	3.03	0.0149	0.0076
M31661	1079_g_at	prolactin (PRL) receptor	3.09	0.0180	0.0231
Z25749	34646_at	ribosomal protein S7	2.95	0.0072	0.0185
AF034176	32218_at	clone ntcon5 contig	2.87	0.0106	0.0285
U90904	38452_at	clone 23773	2.87	0.0104	0.0430
D38048	39060_at	proteasome subunit z	2.80	0.0193	0.0356
AI743134	41246_at	similar to glia derived nexin precursor	2.81	0.0212	0.0383
U66078	33972_r_at	DAZLA	2.58	0.0048	0.0053
AB023154	35369_at	KIAA0937	2.66	0.0072	0.0133
AI540925	41206_r_at	PEC1.2_15_A02.r cDNA	2.26	0.0072	0.0145
U07132	519_g_at	Ner-I steroid hormone receptor	2.29	0.0149	0.0267
AA527880	35774_r_at	NDUFB7	2.38	0.0152	0.0356
AI828210	38592_s_at	IMAGE:2421832	2.35	0.0212	0.0165
U77327	32970_f_at	CD30	1.82	0.0107	0.0211
U40282	35365_at	integrin-linked kinase	1.80	0.0212	0.0154
AF029778	32137_at	jagged 2 (Notch ligand)	1.84	0.0289	0.0422
M92287	1795_g_at	cyclin D3 (CCND3)	1.89	0.0289	0.0193
AF042379	39918_at	GCP2	2.00	0.0107	0.0205
X56468	409_at	14.3.3tau	2.12	0.0152	0.0210

Table 3: Subset of 10 genes from tables 1 and 2 with the most significant differential expression patterns for the pre-CR and the control group. The expression pattern of eight genes are validated by TaqMan™ real-time Q-PCR. nd: not done due to limited sequence information.

accession number	name	description	fold change (HG-U95)	fold change (Q-PCR)
upregulated in preCR group at month 6				
X07696	KRT15	cytoskeletal structural protein	7.96	2.36
M16937	hoxB7	homeodomain family of DNA binding proteins	5.73	2.55
AF040708	NPRL2	candidate tumor suppressor gene 21 protein	4.28	4.60
U41635	OS9	acidic (leucine-rich) nuclear phosphoprotein	2.70	4.20
AB010414	G-protein γ 7	guanine nucleotide binding protein	2.54	4.99
U79251	OBCML	immunoglobulin protein superfamily member	3.48	20.89
AL049449	DKFZp5586B1722	uncharacterized	4.08	4.21
W26469	EST ID 32f4	uncharacterized	2.23	nd
downregulated in preCR group at month 6				
AJ005814	hoxA7	homeodomain transcription factor	-5.03	1.86
M31661	PRLR	prolactin receptor	-3.09	-32.31

Table 4: Sequences and labels of all probes and primers used in TaqMan™ assays.

GenBank accession #	Name	Sequence
X07696	KRT15	5'-GGCTTTGCATGCGCTCTATT-3' 5'-GCTGCATCTCCTTGCTCCA-3' 5'-FAM-CCCCTCTGCCTCTCCCCACCTTC-TAMRA-3'
M16937	HoxB7	5'-GGAGCCCCAAAACCTACCA-3' 5'-AAGCAAGAAGCAGCAGCCA-3' 5'-FAM-TCGCGTGTTCCCCAAGCGC-TAMRA-5'
AF040708	NPR2	5'-TGGGAGTTACCTGAGGGAAGC-3' 5'-GATTGGCAGTGCCCCATG-3' 5'-FAM-AGACCCTTTATGTCTCTCAGGAGCCCTGGA-TAMRA-3'
U41635	OS9	5'-GCAAGGAGGGCAGGACACT-3' 5'-CAAACATCACTAAGGGCAGGTG-3' 5'-FAM-CAGGCACTGAGCAAGCAGGCCC-TAMRA-3'
AB010414	G-protein γ7	5'-TGGCCTTCTCAGTTTGGGC-3' 5'-TTCAGTTATTCCGAACGGGAA-3' 5'-FAM-AAAGGGATGGAGGCTTTACGGCCA-TAMRA-3'
U79251	OBCML	5'-CTGAGCCACCTTTGCTGTCTT-3' 5'-TTTGAATCCCAGGCAACTTTG-3' 5'-FAM-TCTCCTGGGACGAGAAGGACTCATCCA-TAMRA-3'
AL049449	DKFZp586B1722	5'-AACTTGCCAATTCTGTGAATGTTATT-3' 5'-GGGACATGTTACCCAATCACAA-3' 5'-FAM-ATTTAAAAAGCTGGGTCTGTAATGGGAGGCATT-TAMRA-3'
AJ005814	HoxA7	5'-TGGAAATTCTGCTCACTTCTTGC-3' 5-TCTGATGTCATGGCCAAATTTG-3' 5'-FAM-CTTGCTTGCTTCTCTGGTGGGCTTCC-TAMRA-3'
M31661	PRLR	5'-GACACTACTAAAGCTCCCAGCTCC-3' 5'-TTCTGGAATCAGCTGCTGGA-3' 5'-FAM-TTCATGCTCCATTTTTAACCACTTGCCTCTT-TAMRA-3'

Table 5: Demographics of the recipients and donors.

recipient								donor				
patient	age	gender	ethnicity	endstage renal disease	AR	month 12 histology diagnosis	BANFF grading	age	gender	ethnicity	type of tx	HLA mismatch at A-B-DR loci
A	41	F	Ot	GN/GD	1	1	mild	22	M	Ot	CadHB	1-1-2
B	27	M	Ca	GN/GD	0	1	mild	26	F	Ca	LivUnrel	1-2-2
C	42	M	Ca	DM	0	1	mild	39	F	Ca	CadHB	1-1-0
D	62	M	Ca	GN/GD	1	1	mild	53	M	Ca	CadHB	1-1-1
E	54	M	Ca	GN/GD	0	1	mild	44	F	Ca	CadHB	2-1-2
F	50	M	Ca	other	0	1	mild	55	F	Ca	LivRel	1-1-1
G	27	M	Ca	other	0	1	mild	55	F	Ca	Liv rel	1-1-1
H	39	M	Ca	Unkn	0	1	moderate	47	F	Ca	LivUnrel	1-2-2
I	49	F	Ca	PyN/IN	0	1	mild	47	F	Ca	LivUnrel	2-2-1
J	19	F	Bl	Unkn	0	0	none	42	F	Bl	LivRel	1-1-0
K	50	F	Ca	Unkn	0	0	none	23	F	Ca	LivRel	0-0-0
L	53	M	Ca	DM	0	0	none	62	M	Ca	CadHB	1-0-1
M	47	F	Ca	PCKD	1	0	none	51	M	Ot	CadHB	2-0-0
N	30	F	Ot	GN/GD	0	0	none	59	M	Ot	LivRel	0-1-1
O	37	F	Ca	Htn/Nsc	0	0	none	42	F	Ca	LivRel	1-1-1
P	30	M	Bl	Unkn	1	0	none	27	M	Bl	LivRel	2-2-2
Q	21	F	Ot	GN/GD	0	0	none	43	F	Ot	LivRel	0-0-0

Recipients A – I developed CR between month 6 and month 12, patients J – Q remained healthy. AR: number of acute rejection episodes;

Gender: F, female; M, male. Ethnicity: Ot, oriental; Ca, caucasian; Bl, black. End stage renal disease: GN/GD, glomerulonephritis/glomerular disease; PyN/IN, pyelonephritis/interstitial nephritis; PCKD, polycystic kidney disease; Htn/Nsc, hypertension/nephrosclerosis; Vsc, vasculitis; DM = diabetes mellitus; OD/R, obstructive disorder / reflux; Unk, unknown origin. AR: number of acute rejection episodes. Month 12 histology diagnosis: 1, Chronic rejection positive; 0, no rejection. Type of transplant: CadHB, cadaveric heart beating; CadNHB, cadaveric non-heart beating; LivRel, living related; LivUnrel, living unrelated.